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Studies on the Enzymatic Blood-Brain Barrier: Quantitative Measurements of
DOPA Decarboxylase in the Wall of Microvessels as Related to the Parenchyma
in Various CNS Regions

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The presence of DOPA decarboxylase in cerebral microvessels (capillaries and venules) impedes the passage of circulating amine precursors into the brain. The relative amount of DOPA decarboxylase in this trapping mechanism as compared to the parenchyma per se was estimated in various CNS regions, measuring the formation of dopamine from L-DOPA in vitro or in vivo in two experimental models on rats and rabbits: (1) On the basis of the treatment with a peripheral decarboxylase inhibitor (carbidopa, which inhibits also microvascular DOPA decarboxylase in the CNS) it could be calculated that the enzyme in the microvessels of the caudate nucleus (rich in catecholamine nerve terminals), cerebellum (poor in catecholamine nerves), and spinal cord comprised 25, 91, and 79 per cent, respectively, of the total enzyme activity. (2) Measurement of dopamine formation in the spinal cord following transection at the midthoracic level (which causes degeneration of the catecholamine neurons caudal to the lesion since they are all descending) indicated that a similar fraction as found in the carbidopa model, 71 per cent, of the total tissue decarboxylase activity resided in the microvessel walls. The results show that a considerable portion of tissue decarboxylase in the CNS is present in the microvessel walls, where it represents part of an enzymatic blood-brain barrier mechanism.

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The presence of DOPA decarboxylase in cerebral microvessels (capillaries and venules) impedes the passage of circulating amine precursors into the brain. The relative amount of DOPA decarboxylase in this trapping mechanism as compared to the parenchyma per se was estimated in various CNS regions, measuring the formation of dopamine from [3-DOPA] *in vivo* or *in vitro* in two experimental models on rats and rabbits: (1) On the basis of the treatment with a peripheral decarboxylase inhibitor (carbidopa), which inhibits the microvessel DOPA decarboxylase in the CNS it could be calculated that the enzyme in the microvessels of the cerebral nucleus (rich in catecholamine nerve terminals), cerebellum (poor in catecholamine nerves), and spinal cord comprised 25, 91, and 73 per cent, respectively, of the total enzyme activity. (2) Measurement of dopamine formation in the spinal cord following the injection of [3-DOPA] into the cerebrospinal fluid indicated that catecholamine nerves (rich in the enzyme) are of all descending) indicated that a similar fraction is found in the cerebrospinal fluid, 77 per cent of the total tissue decarboxylase activity resided in the microvessel walls. The results show that a considerable portion of tissue decarboxylase in the CNS is present in the microvessel walls, where it represents part of an enzymatic blood-brain barrier mechanism.

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The concept of a barrier between the blood and the brain parenchyma originates from the observation by Ehrlich (1885) that vital dyes - when injected systemically - pass freely from the circulation into peripheral tissues, but are excluded from the central nervous system. With the advent of electron microscopy an ultrastructural basis for the blood-brain barrier was recognized in that cerebral vessels are distinguished by the presence of closed tight junctions between adjacent endothelial cells (Reese and Karnovsky, 1967). This feature - unique for the cerebral vasculature - along with absence of endothelial fenestrae and the paucity of pinocytotic vesicular transport across the endothelial cells, constitutes the morphological basis for the blood-brain barrier. In this way the diffusion of substances from the systemic circulation into the brain is severely limited (for review, see Rapoport, 1976).

Using fluorometric assay and fluorescence microscopic techniques, a number of histochemical investigations have revealed that there is a specific trapping mechanism located within the cerebral capillary endothelium for amine precursors, such as L-DOPA (L-3,4-dihydroxyphenylalanine) and L-5-hydroxytryptophan (Bertler et al., 1966; Owman and Rosengren, 1971; Bartholini et al., 1971; Hardebo et al., 1976). This trapping mechanism, which constitutes part of an enzymatic barrier, has essentially three phases: the active transport (Wade and Katzman, 1975) of the precursor into the endothelial cell; the decarboxylation of the precursor to the corresponding amine; and finally, the amines are metabolized by the enzyme monoamine oxidase.

The degree of decarboxylation capacity can be measured directly in isolated fractions of brain microvessels which are essentially free of neuronal contamination (Hardebo et al., 1977 a). The capacity of the enzymatic blood-brain barrier under in vivo conditions has been estimated in terms of the break-through of L-DOPA into the brain after systemic administration of increasing doses of the amino acid (Hardebo et al., 1977 b). The present paper offers further quantitative aspects of the microvascular trapping mechanism for L-DOPA in the brain by chemical determinations of the DOPA decarboxylase activity in various CNS regions using two models: either after elimination of the neurons (caudal to a spinal cord transection), or following selective inhibition of the enzyme in the vessels (by carbidopa which, at certain dose levels, efficiently blocks DOPA decarboxylase in peripheral tissue and brain microvessels, leaving the enzyme within the brain parenchyma essentially unaffected; Bartholini and Pletscher, 1969).

Material and Methods

Animals. The study was performed on 16 female Sprague-Dawley rats, weighing 190–200 g, and 18 female albino rabbits weighing 1.5 to 2.0 kg. The animals had free access to standard pellet food and tap water. The spinal cord in 11 of the rats and in all rabbits was transected in the midthoracic region under brietal sodium anesthesia. The urinary bladder was emptied daily by gentle pressure on the abdomen, and care was taken to prevent deformation of the extremities. The animals were killed under light ether anesthesia 10 days after transection by perfusion of the vascular system with 0.9 % saline.

Fluorescence microscopy. Immediately after decapitation, small tissue pieces were dissected out from the cerebellar hemispheres, the caudate nucleus, and the spinal cord. They were frozen to the temperature of liquid nitrogen and further processed for monoamine fluorescence histochemistry according to the Falck-Hillarp method (Björklund, Falck and Owman 1972). The paraformaldehyde used had previously been equilibrated with air of 70 % of humidity. The formaldehyde-induced, histochemically visible fluorophores of noradrenaline (NA), dopamine (DA), and DOPA are indistinguishable under standard conditions since they have the same spectral characteristics and all exhibit a green light under the optical conditions used (Björklund et al. 1972).

Chemical determinations of catecholamines. DA was determined fluorometrically either according to Häggendal (1963) or Anton and Sayre (1964) following homogenization of the tissue in 0.4 N perchloric acid. NA was estimated by the radioenzymatic method described by Cuello, Hiley and Iversen (1973) as modified by Coyle and Henry (1973); the tissue was homogenized in 0.1 N perchloric acid containing 0.1 mg/ml EDTA.

Determination of DOPA decarboxylase activity. (1) One mm thin slices of the cerebellum, caudate nucleus and cervical spinal cord were cut with a razor blade and transferred to incubation vials containing ice-cold Krebs-Ringer buffer solution. The buffer had the following composition (mM): NaCl 118, KCl 4.5, $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ 2.5, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ 1.0, NaHCO_3 25, KH_2PO_4 1.0 to which was added 1 mg/ml glucose, 0.2 mg/ml ascorbic acid and 0.1 mg/ml EDTA. The vials were placed in an incubation bath at 37° C for pre-incubation during 10 min followed by incubation during 20 min in the presence of 0.1 mg/ml L-DOPA . Blanks were incubated without L-DOPA . The buffer solution was continuously aerated with a mixture of 95 % O_2 and 5 % CO_2 giving a pH of 7.4. One slice from each incubation was taken for fluorescence microscopy and the remainder for chemical determination of DA according to Häggendal (1963).

(2) Tissue pieces from the spinal cord were homogenized on ice and preincubated for 10 min in 67 mM phosphate buffer (pH 7.5) to which pyridoxal -5'-phosphate had been added, followed by incubation for 45 min in the presence of 0.1 mg/ml L-DOPA . The in-

cubation was performed at 37° C under anoxic conditions (continuous bubbling with nitrogen; Bertler and Rosengren 1959). The incubation was stopped with 0.4 N perchloric acid. Boiled homogenate incubated in L-DOPA served as blank. The amount of DA formed was determined according to Häggendal (1963).

There was no statistically significant difference (Student's t-test) whether the dopamine formation was determined under anoxic conditions or not, even with extended incubation times from 20 to 45 min. In the Results it is therefore not specified which of the two techniques that was used in the DOPA decarboxylase determinations.

Experimental. Three types of experiments were performed with animals in which the spinal cord had been transected:

(1) Three rats and 8 rabbits were pretreated with nialamide (100 mg/kg i.p.) 90 min before the injection of L-DOPA (50 mg/kg i.p.). All rats and 6 rabbits were killed 20 min later, whereas 2 rabbits were killed after 60 min. The spinal cord was dissected out and pieces were taken cranial and caudal to the lesion for chemical determination of DA.

(2) Three rats and 4 rabbits were pretreated with nialamide (100 mg/kg i.p.) 90 min before sacrifice. One mm thin slices of the spinal cord were taken above and below the level of transection for determination of DOPA decarboxylase activity under normoxic conditions.

(3) Spinal cord tissue above and below the lesion was homogenized on ice for determination of DOPA decarboxylase activity under anoxic conditions (3 rats and 4 rabbits) or homogenized in perchloric acid for determination of NA (2 rats and 2 rabbits).

Experiments with DOPA decarboxylase inhibition were carried out with intact rats: 5 rats were pretreated with reserpine (10 mg/kg i.p.) and 4 hrs later with nialamide (100 mg/kg i.p.), followed after 30 min by carbidopa at a dose (100 mg/kg i.p.) which effectively inhibits DOPA decarboxylase in peripheral tissue and brain microvessels (leaving the decarboxylase activity within the brain parenchyma essentially unaffected; Bartholini and Pletscher 1969). The animals were killed 1 hr after the last injection. Five animals received reserpine and nialamide, but no carbidopa. Slices from the cerebellum, caudate nucleus, and spinal cord were incubated under normoxic conditions for determination of DOPA decarboxylase activity.

Drugs. The following compounds were used: L-3,4 dihydroxyphenylalanine (Sigma), reserpine (Serpasil, Ciba), nialamide (Niamid, Pfizer), α -methyldopa hydrazine (Carbidopa, MSD), pyridoxal-5'-phosphate (British Drug House), brietal sodium (Lilly).

Results and Comments

Fluorescence microscopy of the caudate nucleus from animals treated with reserpine and nialamide with or without the peripheral decarboxylase inhibitor, carbidopa, followed by incubation with $\underline{\underline{L}}$ -DOPA showed an intense green fluorescence in the parenchyma deriving from the very dense network of dopamine-containing axon terminals (cf. Lindvall and Björklund 1974). The general background fluorescence of the parenchyma in the cerebellum and spinal cord was very low, and only a small number of isolated green-fluorescent axons known to contain NE (Olson and Fuxe 1971, Pickel, Segal and Bloom 1974, Nygren and Olson 1977) was present in the preparations.

In animals not given carbidopa, a green fluorescence of equally high intensity was seen in the walls of capillaries as well as of small veins in all regions studied (Fig. 1), whereas the walls of parenchymal and pial arteries and arterioles were non-fluorescent. The microvessel fluorescence was weaker in the rabbits (killed 20 or 60 min after administration of $\underline{\underline{L}}$ -DOPA) than in the rats (killed 20 min after $\underline{\underline{L}}$ -DOPA). Tissues from animals pretreated with carbidopa (following reserpine and nialamide as above) and incubated with $\underline{\underline{L}}$ -DOPA showed only a weak fluorescence in the microvessel wall but, on the other hand, an increased diffuse background fluorescence of the parenchyma.

The density of the network of microvessels was compared in the three regions on the basis of the arrangement of intensely fluorescent vessel walls. The density was lower in white than in grey matter, but no difference in density was found between the various regions.

Microvessel DOPA decarboxylase activity (carbidopa model). An estimate of the DOPA decarboxylase activity in caudate, cerebellar, and spinal cord microvessels was obtained by subtracting the tissue decarboxylase value in animals treated with carbidopa (and thus having only central neuronal decarboxylase intact) from the value of animals not given carbidopa (having both neuronal and vascular decarboxylase). Values obtained during incubation without $\underline{\underline{L}}$ -DOPA (representing endogenous DA) were subtracted beforehand. The vascular decarboxylase activity thus calculated was in the cerebellum and spinal cord 2.82 ± 0.50 and 3.12 ± 0.83 $\mu\text{g DA/g tissue} \times 20 \text{ min}$, respectively (mean values $\pm$ S.E.M.; Table 1). In the caudate nucleus the mean activity was slightly higher, 4.72 ± 0.78 $\text{mg DA/g} \times 10 \text{ min}$. The relative vascular activity comprised 25, 91, and 79 per cent of the total (neuronal plus vascular) DOPA decarboxylase activity in the caudate nucleus, cerebellum and spinal cord, respectively. Since the cerebellum and spinal cord contain only few catecholamine-forming neurons it is to be anticipated that a major fraction of the total decarboxylase activity in these regions is represented by the microvessel walls. The situation reverse in the caudate nucleus, rich in dopaminergic nerve terminals.

In untreated animals the NA concentration in the spinal cord is about the same in segments cranial and caudal to the midthoracic level (Andén, Magnusson and Rosengren 1965). Ten days after thoracic transection the concentration caudal to the lesion dropped to 15 ± 2 and 10 ± 2 per cent of the concentration cranial to the section in the rat and rabbit, respectively.

Microvessel DOPA decarboxylase activity (spinal cord transection model). The degree of DA formation obtained in vivo and in vitro cranial and caudal to the midthoracic transection in the rat and rabbit are shown in Fig. 2. In non-operated animals, the DA formation is about the same cranial and caudal to the transection level (Andén et al. 1965). However, after transection the decarboxylase activity in the cranial portion does not differ significantly from the activity in the corresponding region of non-operated animals (Andén et al. 1965). In the present study, a mean fall in activity of 29 per cent was seen in the rat after transection, i.e. the DOPA decarboxylase activity present in microvessel walls (and in perivascular sympathetic nerves) would constitute about 70 per cent of the total activity in the spinal cord (cf. the value calculated after vascular decarboxylase inhibition). The corresponding value in the rabbit was considerably lower, about 45 per cent.

Discussion

The mechanism that involves decarboxylation and subsequent degradation of circulating monoamine precursors at the level of the blood-brain barrier (Bertler et al. 1966, Hardebo et al. 1976) is of interest both from a general biological point of view (see Rapoport 1976) and clinically in the medical treatment of Parkinson's disease (Cotzias, Papavasiliou and Gellene 1969). It has previously been shown that the decarboxylation capacity in the wall of brain microvessels has a limit (Hardebo et al. 1977b), which means that the amine precursor (e.g. L-DOPA) is able to penetrate into the brain parenchyma at particularly high circulating concentrations. This limit was estimated in a model where increasing doses of L-DOPA were given systemically to rats, and the break-through of L-DOPA into the brain parenchyma via the enzymatic blood-brain barrier was estimated by comparing the amount of newly formed DA in brain structures with and without decarboxylase-containing neurons (the caudate nucleus and cerebellum, respectively). The microvessels were found to efficiently trap L-DOPA in their walls up to a circulating level corresponding to an i.p. dose of 100 mg/kg. It could be estimated that approximately 3 per cent of the exogenous L-DOPA is decarboxylated at the level of the blood-brain barrier, whereas the remainder of the conversion takes place in peripheral tissues (Hardebo et al. 1977b).

In the present study the efficiency of the trapping mechanism was investigated more directly by measuring the decarboxylating capacity in the microvessel wall of the cerebrospinal parenchyma. The major problem encountered was to separate that portion of the decarboxylation taking place in the microvessels from the amine formation occurring in the monoaminergic neuron systems. Two approaches were used: one in which the microvascular, but not neuronal, decarboxylase was inactivated by carbidopa and another in which the neurons were selectively eliminated by transection of the spinal cord, leaving the microvascular amine-forming capacity intact.

The decarboxylase inhibitor, carbidopa (α -methyldopa hydrazine), is selective in the sense that it, probably due to a poor penetration through the blood-brain barrier, leaves neuronal decarboxylase in the central nervous system unaffected at certain dose levels which only inhibits peripheral DOPA decarboxylase, including that in the brain microvessel walls (Bartholini and Pletscher 1969). The effect of carbidopa was checked by fluorescence microscopy. Thus, incubation of brain tissue in the presence of L-DOPA produced an intense fluorescence (which has been shown to be represented mainly by dopamine; Bertler et al. 1966) in the microvessel walls that, on the other hand, was almost absent in tissues from carbidopa-treated animals. Persisting decarboxylase activity in the neuron system (of reserpine-pretreated animals) was indicated by an equally high neuronal fluorescence after the L-DOPA incubation, whether the animals had received carbidopa or not. It was therefore assumed that most — if not all — of the decarboxylase activity measured in preparations from carbidopa-treated animals reflected neuronal enzyme activity. This was corroborated by the finding that the neuronal decarboxylase thus calculated comprised a much higher fraction of total decarboxylase in the caudate nucleus than in the cerebellum and spinal cord, which contain a much lower number of catecholaminergic neurons. In fact, the relationship between the decarboxylase activity measured in the brain parenchyma of carbidopa-treated animals corresponds very well to the relative concentrations of catecholamines in the spinal cord, cerebellum and caudate nucleus, respectively (see catecholamine figures compiled by Holzbauer and Sharman 1972).

The monoamine neuron system in the spinal cord is entirely descending, which means that transection produces an almost complete disappearance of NA caudal to the lesion (Andén et al. 1965). It can thus be predicted that remaining catecholamine formation occurs in the intraspinal vessel walls (and probably, to a minor extent, in perivascular adrenergic nerves arriving to the spinal cord segmentally). After transection, the DOPA decarboxylase activity in the caudal portion of the cord dropped by 29 % in the rat, which

would indicate that approximately 70 % of spinal cord DOPA decarboxylase activity resides in the vascular system. This figure agrees well with the value (79%) calculated from the carbidopa model.

The corresponding figure for the intraspinal vascular DOPA decarboxylase activity in the rabbit was lower, only 45 %, compared with the rat values. This is in good agreement with the lower enzyme activity that is reflected by a lower wall fluorescence in the microvessels of rabbits under conditions equal to those for rats. The corresponding figure in rabbit experiments presented by Andén et al. (1965) are somewhat lower.

In the previously mentioned in vivo model, in which the decarboxylating capacity in the microvessel walls was estimated after i.p. administration of increasing doses of L-DOPA (to animals pretreated with reserpine and the monoamine oxidase inhibitor nialamide), it was found that the maximum amount of DA formed in the cerebellar vessels corresponded to a tissue concentration of 3.12 µg/g during 20 min. The correlating value for the in vitro formation calculated from the carbidopa model (see Table I) was very close, 2.82 µg/g formed during 20 min, showing a good agreement between the two different model approaches. A regional difference was found with the carbidopa model, in terms of microvascular DA formation, being in the order caudate nucleus > spinal cord > cerebellum. The same order of difference has been found when comparing the decarboxylase activity in isolated fractions of microvessels from the caudate nucleus and cerebellum in various animals (Hardebo et al., 1978), and it was also indicated by the cytofluorometric measurements of the formaldehyde-induced capillary wall fluorescence in rats given L-DOPA (Hardebo et al., 1977 b).

Particularly high doses of L-DOPA have been given in the medical treatment of Parkinson's disease due to the decarboxylation activity in the peripheral tissues and the efficiency of the blood-brain barrier to impede the passage of L-DOPA into the brain, with frequent side-effects as result. Based on this knowledge, including the decarboxylating capacity of the blood-brain barrier (Bertler et al., 1966, Bartholini et al., 1971), it has now been possible to overcome this problem: a decarboxylase inhibitor, effective in peripheral tissues as well as in brain capillary walls but not in the brain parenchyma, has been added to L-DOPA in the drug treatment of Parkinson's disease. This means that the amine precursor can now be administered in lower doses, reducing the incidence of side-effects.

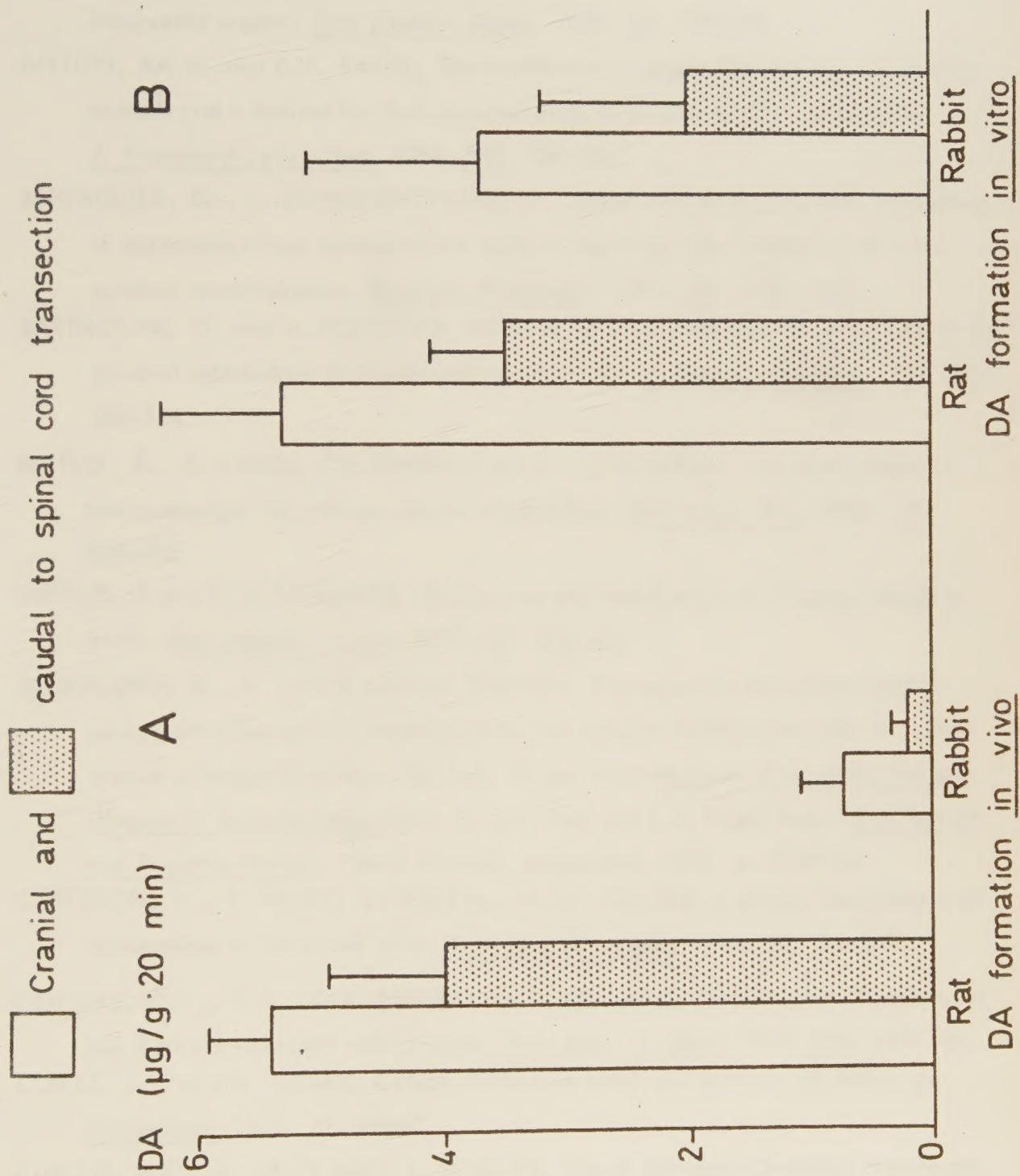
Acknowledgement: This study was supported by the Swedish Medical Research Council (Grants No. 04X-732 and 04X-56).

Fig. 1 Spinal cord from a rat pretreated with 10 mg/kg reserpine followed by 100 mg/kg nialamide. Twenty min after the injection of 50 mg/kg L-DOPA an intense green fluorescence is seen in the microvessel walls. There is essentially no background fluorescence in the parenchyma. Those of the nuclear regions that are intact in the tissue section represent the largest volume of the pericytes and endothelial cells and are therefore particularly well visible. X 290.

Fig. 2 DOPA decarboxylase activity (mean values + S. E. M.) expressed as μ g dopamine (DA) formed per g tissue (wet weight) cranial and caudal to spinal cord transection in the rat and rabbit. A: Formation in vivo, animals treated with nialamide (100 mg/kg i.p.) 1 h followed by L-DOPA (50 mg/kg i.p.) 20 min. B: Formation in vitro, tissue slices preincubated in nialamide (10^{-3} M) 20 min, followed by incubation in L-DOPA (0.1 mg/ml) 20 min.



Fig. 1



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